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Isolation and characterization of brown algal polyphenols as inhibitors of α-amylase, lipase and trypsin

Clive J. Barwell, Gerald Blunden & Prabha D. Manandhar
School of Pharmacy & Biomedical Sciences, Portsmouth Polytechnic, King Henry I Street, Portsmouth, Hampshire PO1 2DZ, U.K.

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Abstract

Extracts of *Ascophyllum nodosum*, *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* contain inhibitors of α-amylase, lipase and trypsin. The inhibitors were isolated and identified by ^{1}H NMR spectroscopy as polyphenols which have apparent molecular weights in the range from 30000 to 100000 daltons, as determined by ultra-filtration with Amicon membranes. These polyphenols account for the whole of the inhibitory activity in crude algal extracts. The compounds inhibit α-amylase and trypsin in an apparently non-competitive manner, when preincubated with the enzymes, and the inhibition is directly proportional to the concentration of the inhibitor. Starch protects α-amylase when added to the enzyme together with the inhibitors. Under this condition the effectiveness of the inhibitors is reduced ten-fold.

Introduction

As part of a survey of British marine algae for compounds with biological activity, extracts of thirty-six species were tested for the presence of inhibitors of α-amylase. Only two species gave positive results and of these the highest activity occurred in extracts of *Ascophyllum nodosum* (L.) Le Jol. (Barwell *et al.*, 1981). As this alga is used in both animal feedstuffs and in human 'health foods' it was considered of interest to investigate further the nature of the enzyme inhibitory activity in extracts of *A. nodosum*. In addition, some other brown algae which commonly occur in either the same or similar habitats, and therefore may be harvested together with *A. nodosum*, were investigated.

Materials and methods

Laminaria digitata (Huds.) Lamour., *L. hyperborea* (Gunn.) Fosl., *Ascophyllum nodosum* (L.) Le Jol, *Fucus serratus* L., *F. vesiculosus* L., and *Pelvetia canaliculata* (L.) Dcne et Thur., were collected at Finavarra, County Clare, Ireland, in September 1982.

Extraction of algae and isolation of enzyme inhibitors

Algae were dried at 60 °C and powdered. Dry alga (5 g) was extracted with ethanol in a Soxhlet apparatus for 6 h and the extract evaporated *in vacuo* at 50 °C. The residue was extracted with

20 ml water and the extract clarified by centrifugation. This aqueous extract was used to determine total inhibitory activity. For isolation of inhibitors, the extract was dialysed against water and the non-dialysable fraction freeze-dried. The solids obtained were then fractionated into an ethyl acetate and acetone soluble fraction as described by Geiselman & McConnell (1981). ^{1}H NMR spectra were obtained in acetone d_6 using a Brüker WH-270 spectrometer.

Assay of enzymes and inhibitors

α-Amylase (EC 3.2.1.1.), Sigma type VII-A, was assayed at pH 7 and 37 °C, with 2 μg enzyme protein and 10 mg ml^{-1} soluble starch, as described by Robyt & Whelan (1968), using maltose as the standard reducing sugar. Lipase (EC 3.1.1.3.), Sigma type VI, was assayed at pH 8.0 and 37 °C, with 50 μg enzyme protein and 25% (v/v) olive oil, as described for the lipase assay kit No. 800, supplied by Sigma London Ltd., Poole, UK. Trypsin (EC 3.4.21.4.), Sigma type III, was assayed at pH 8.2 and 37 °C, with 10 μg enzyme protein and 1 mM N-α-benzoyl-DL-arginine p-nitroanilide hydrochloride, as described by Erlanger *et al.* (1961).

For the assay of inhibitors, enzyme was preincubated, in the absence and presence of inhibitor, for 5 min at 37 °C, after which the substrate was added. Preliminary experiments established that, in the absence of inhibitor, there was no loss of enzyme activity. Determinations of enzyme activity were made within the range where there was a linear relationship between the measured activity and concentration of enzyme. One inhibitor unit was contained in the volume of inhibitor solution which gave 50% inhibition of enzyme activity. Results are given as mean ± s.e. mean with the number of observations. Kinetic experiments were carried out by measuring initial velocities (v) with five substrate concentrations (s), ranging from one quarter to four times the apparent Michaelis constant. Kinetic constants were obtained from Hanes plots (s/v against s) in which lines of best fit were obtained by linear regression.

Results

Inhibitory activity of algal extracts

Table 1 shows that extracts of *Ascophyllum nodosum*, *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* inhibited α-amylase, lipase and trypsin. In contrast, extracts of *Laminaria digitata* and *L. hyperborea* did not inhibit these enzymes.

Table 2 shows that the compounds responsible for the inhibitory activity towards α-amylase, extracted from *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*, were retained within dialysis tubing. All of the inhibitory compounds (102 ± 5%, $n = 3$) in an extract of *A. nodosum* passed through an Amicon 100A membrane whilst they were all (97 ± 6%, $n = 3$) retained by an Amicon PM30 membrane. Similar results were obtained for inhibitor activity towards lipase and

Table 1. Inhibitor activity towards α-amylase, lipase and trypsin in extracts of some brown algae. One inhibitor unit gave 50% inhibition of enzyme activity under conditions described in Materials and methods.

Algal species	Inhibitor units per g dry weight algae towards:		
	α-amylase	Lipase	Trypsin
A. nodosum	40000	1400	8000
F. serratus	40000	1800	16000
F. vesiculosus	80000	3000	16000
P. canaliculata	40000	1400	8000
L. digitata	0	0	0
L. hyperborea	0	0	0

Table 2. Effect of dialysis upon the inhibitor activity towards α-amylase. One inhibitor unit gave 50% inhibition of enzyme activity under conditions described in Materials and methods. Values are mean ± s.e., $n = 4$.

Algal species	α-amylase inhibitor units in:	
	Non-dialysed extract	Dialysed extract
A. nodosum	8300 ± 400	9400 ± 300
F. serratus	10200 ± 500	10000 ± 500
F. vesiculosus	12500 ± 300	12500 ± 500
P. canaliculata	9000 ± 350	8700 ± 300

trypsin, and for the inhibitor activity of extracts of *F. serratus*, *F. vesiculosus* and *P. canaliculata* towards α-amylase, lipase and trypsin.

Isolation of inhibitors

Ethyl acetate and acetone fractions of the non-dialysable solids, isolated from *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*, yielded light-brown solids which were freely soluble in water. The ^{1}H NMR spectra of the two fractions, from each alga, were essentially the same and exhibited resonances from δ5.67 to δ6.31, which closely matched those reported by Geiselman & McConnell (1981) for non-dialysable polyphenols isolated from *A. nodosum* and *F. vesiculosus*. The nature of the spectra indicated that the polyphenols constituted the great majority of the isolated material. Inhibitory activity towards α-amylase, lipase and trypsin was exhibited by the material in both the ethyl acetate and acetone fraction from *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*. Table 3 shows results obtained with *A. nodosum*. It can be seen that the majority of the inhibitory activity, exhibited by the algal extract, was recovered as the sum of the activity in the ethyl acetate and acetone fractions. In contrast, the ethyl acetate and acetone fractions of the non-dialysable solids from both *L. digitata* and *L. hyperborea* were only very small, did not exhibit resonances in the region from δ5.0 to δ7.0 in their ^{1}H NMR spectra and also did not inhibit any of the three enzymes.

Table 3. Inhibitory activity towards α-amylase, lipase and trypsin in an extract of *A. nodosum* and the ethyl acetate and acetone fractions derived from it. One inhibitor unit gave 50% inhibition of enzyme activity under conditions described in Materials and methods. Values are mean ± s.e. of 4 determinations upon fractions from one isolation procedure.

Fraction	Inhibitor units per g dry weight alga towards:		
	α-amylase	Lipase	Trypsin
Dialysed extract	40000 ± 900	1400 ± 50	8000 ± 300
Ethyl acetate	33000 ± 200	780 ± 20	6600 ± 180
Acetone	7700 ± 500	470 ± 30	1800 ± 100
Ethyl acetate + acetone	40700	1250	8400

Characterization of the enzyme inhibition

α-Amylase, lipase and trypsin were preincubated with inhibitor, isolated from *A. nodosum*, for times which ranged from 1 to 10 min. Maximum inhibition of each enzyme occurred within 1 min. With a standard preincubation time of 5 min, the percentage inhibition of each enzyme was directly proportional to the amount of inhibitor present. When the inhibition was calculated in terms of enzyme-protein inhibited, by inhibitors from 1 g dry weight of algae, similar values were obtained. The values obtained, with inhibitor isolated from *A. nodosum*, were; 40 ± 1 mg for α-amylase, 35 ± 1 mg for lipase and 40.0 ± 2 mg for trypsin (n = 4).

Hanes plots for α-amylase and trypsin activity, in the absence and presence of inhibitor from *A. nodosum*, were characteristic of non-competitive inhibition, in that the inhibitor reduced the maximum velocity whilst the Michaelis constant was not altered. With α-amylase, maximum velocities were 193 μM min^{-1} in the absence and 96 μM min^{-1} in the presence of inhibitor, whilst the Michaelis constant was 320 μg ml^{-1} in both conditions. With trypsin, maximum velocities were 7.5 μM min^{-1} in the absence and 2.6 μM min^{-1} in the presence of inhibitor, whilst the Michaelis constants were 780 μM and 770 μM, respectively. The possibility that starch might protect α-amylase from inhibition was investigated with inhibitor from *A. nodosum*. An amount which gave 90% inhibition, when preincubated with α-amylase, produced no inhibition when it was added to the enzyme at the same time as the substrate. Under these latter conditions, the amount of inhibitor required to produce 50% inhibition was ten times greater than when the enzyme was preincubated with inhibitor.

Discussion

Results of this investigation confirmed the original observation of Barwell *et al.* (1981) that extracts of *Ascophyllum nodosum* inhibit α-amylase. In addition, the present study showed that lipase and trypsin are inhibited and that extracts of *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* also inhibit these enzymes. The inhibitors were non-dialysable. Since non-dialysable polyphenols, which could act as enzyme inhibitors (Loomis, 1966), occur in brown algae, the procedure used by Geiselman & McConnell (1981) to isolate non-dialysable polyphenols from *A. nodosum* and *F. vesiculosus* was applied to our inhibitor-containing extracts. The ^{1}H NMR spectra of the non-dialysable compounds isolated by us closely matched those recorded by Geiselman & McConnell (1981) and the spectra indicated that the isolated solids were composed almost entirely of phenolic compounds. The inhibitory activity of the dialysed extracts was quantitatively maintained in the polyphenol-containing fractions. Therefore, it appears that the inhibitor activity, in extracts of *A. nodosum*, *F. serratus*, *F. vesiculosus* and *P. canaliculata*, is due to their content of non-dialysable polyphenols. These behaved, during ultra-filtration, like spherical molecules with molecular weights greater than 30000 and less than 100000 Daltons. The brown algal polyphenols are composed predominantly of phloroglucinol units and can occur as branched structures (Ragan & Jensen, 1977). However, they are not spherical molecules and, therefore, their behaviour during ultra-filtration would not accurately indicate their molecular weights.

Extracts of *Laminaria digitata* and *L. hyperborea* did not inhibit α-amylase, lipase and trypsin and did not yield detectable amounts of non-dialysable polyphenols. Polyphenols have been reported in *Laminaria* species but, when present, occurred at much lower levels than in species of the Fucaceae (Ragan & Jensen, 1977).

The polyphenols isolated in this study inhibited each of the three mammalian digestive enzymes tested. Kinetic experiments with α-amylase and trypsin demonstrated that the inhibitors reduced the maximum velocity but did not alter the Michaelis constant. This effect is obtained with both non-competitive reversible inhibitors and irreversible inhibitors. The essentially linear relationship between percent inhibition and inhibitor concentration showed that the inhibition could not be of the reversible type, which would yield a hyperbolic relationship. Under the experimental conditions used here, polyphenols would react with protein by formation of hydrogen bonds with carbonyl groups of the proteins' peptide bonds. A high molecular weight polyphenol could form large numbers of hydrogen bonds with a protein molecule, resulting in a stable complex and apparently irreversible inhibition (Loomis, 1974). The fact that a particular amount of the isolated polyphenol inhibited essentially the same amount of α-amylase, lipase and trypsin protein demonstrated the non-specific nature of the interaction between the polyphenols and enzymes used here. Indeed, the polyphenols may be expected to inhibit most enzymes and react with most other proteins (Loomis, 1974). The non-dialysable polyphenols from *A. nodosum* and some other brown algae have been shown to cause non-specific aggregation of human erythrocytes (Rogers & Loveless, 1985; Blunden *et al.*, 1986), presumably because one polyphenol molecule can react with protein at the cell surface of several erythrocytes.

A. nodosum, *F. serratus*, *F. vesiculosus* and *P. canaliculata* can be incorporated into both animal feed-stuffs and human 'health foods'. The inhibitory effect of their polyphenols upon mammalian digestive enzymes raises the question of their suitability. The non-specific nature of the interaction of polyphenols with proteins means that they will interact with dietary protein so that their inhibitory effect upon digestive enzymes would be less than that predicted by *in vitro* tests with a single enzyme. Probably, the amount consumed in 'health foods' is negligible with regard to enzyme inhibition. Supplementation of the feed of pigs and sheep with brown algal meals can enhance weight gain (Homb, 1961). However, there is a relatively low limit to the amount of supplement which may be incorporated before

weight gain becomes less than with unsupplemented feed. This may be due to inhibition of digestive enzymes by algal polyphenols resulting in impaired digestion of the bulk of the feed's constituents. Below these levels seaweed meal supplements probably fulfill the role for which they are intended, that is as a source of vitamins and minerals (Guiry & Blunden, 1980).

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